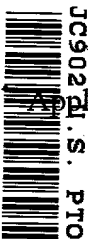


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Title: **USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND ANALOGS THEREOF FOR INDUCING CYTOKINES**

Commissioner for Patents
 ATTN: Box Patent Application
 Washington, D.C. 20231

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- ☒ Utility Patent Application: Specification (33 pgs); Claims (36 claims on 8 pgs); 1 pg Abstract; and Sequence Listing 6 pgs.)
☐ Design Patent Application: _ pgs.
☐ Sheet(s) of drawing(s).
☒ Communication regarding sequence listing (1 page)
☒ Computer Readable Form of Sequence Listing (1 diskette)
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	Number of Claims Filed (1)	Claims Included in Basic Filing Fee (2)	Number of Extra Claims (1-2)	Cost per Extra Claim	Fee Required
Total Claims	36	- 20 =	16	x \$18 =	288
Independent Claims	6	- 3 =	3	x \$78 =	234
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**USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND
ANALOGS THEREOF FOR INDUCING CYTOKINES**

5 **Cross-Reference to Related Applications**

The present application claims priority from U.S. Patent Application
Serial No. 60/149,311, filed on August 17, 1999, which is incorporated herein by
reference.

10 **Background of the Invention**

Colostrum is a component of the milk of mammals during the first few
days after birth. Colostrum is a thick yellowish fluid and is the first lacteal
secretion post parturition and contains a high concentration of immunogloblins
(IgG, IgM, and IgA) and a variety of non-specific proteins. Colostrum also
15 contains various cells such as granular and stromal cells, neutrophils,
monocyte/macrophages, and lymphocytes. Colostrum also includes growth
factors, hormones, and cytokines. Unlike mature breast milk, colostrum
contains low sugar, low iron, but is rich in lipids, proteins, mineral salts,
vitamins, and immunoglobins.

20 Colostrum also includes or contains a proline-rich polypeptide aggregate,
which is referred to as colostrinin. One peptide fragment of colostrinin is Val-
Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (SEQ ID NO:31), which is disclosed in
International Publication No. WO-A-98/14473. Colostrinin and this fragment
have been identified as useful in the treatment of disorders of the central nervous
25 system, neurological disorders, mental disorders, dementia, neurodegenerative
diseases, Alzheimer's disease, motor neurone disease, psychosis, neurosis,
chronic disorders of the immune system, diseases with a bacterial and viral
aetiology, and acquired immunological deficiencies as set forth in International
Publication No. WO-A-98/14473.

30 Although certain uses for colostrinin have been identified, it would

represent an advancement in the art to discover and disclose other uses for colostrinin, or a component thereof, that are not readily ascertainable from the information currently known about colostrinin or its constituents.

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SUMMARY OF THE INVENTION

The present invention relates to the use of colostrinin, at least one constituent (i.e., component) peptide thereof, at least one active analog thereof (e.g., peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides), and combinations thereof, as a cytokine-inducing agent. These agents can be used as immunological regulators to modulate (e.g., enhance, inhibit, modify, augment, or otherwise alter, and preferably promote) specific or nonspecific immune responses in patients, particularly animals including mammals such as humans. They can also be used as blood cell regulators to modulate (e.g., enhance, inhibit, modify, augment, or otherwise alter, preferably, and promote) cellular proliferation or differentiation (preferably, promoting proliferation and differentiation) of blood cells, such as leukocytes.

In one embodiment, the present invention provides a method of inducing a cytokine in a cell. The method includes contacting the cell with an immunological regulator under conditions effective to induce (i.e., induce the synthesis or production of) at least one cytokine (either directly or indirectly), wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLQPFQVQS (SEQ ID NO:3); LFFFLPVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPPF (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIP (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ

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an analog thereof, and combinations thereof, under conditions effective to change the number of blood cells. The blood cells can be in a cell culture or an organism. Hence, this method can be carried out *in vivo* or *in vitro*.

In still another embodiment, there is provided a method for modulating blood cell proliferation in a patient (preferably, a human patient). The method includes administering to the patient a blood cell regulator selected from the group of colostrinin, a constituent peptide thereof, an analog thereof, and combinations thereof, under conditions effective to change the number of blood cells.

The blood cells can be mammalian blood cells, such as human blood cells. Preferably, the blood cells are increased in number, although a decrease in number can also be desirable in certain situations such as leukemia, myelopathy, etc. More preferably, the blood cells are increased in number and differentiated. The blood cell regulator is preferably a constituent peptide of colostrinin.

In other embodiments, the invention provides the use of an immunological regulator or blood cell regulator in the manufacture of a medicament for use in the methods described herein.

The present invention also provides an immune-inducing composition that includes a pharmaceutical carrier and an active agent selected from the

MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2);
DQPPDVEKPDLPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID
NO:4); DLEMPVLPVEPFPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID
NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPPFKLKVEVFPFP (SEQ
ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID
NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ
ID NO:14); SEEMP (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16);
VLPPNVG (SEQ ID NO:17); VYPFTGPIPN (SEQ ID NO:18); SLPQNILPL
(SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20);
LQPEIMGVVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKYPVEPFTESQ
(SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP
(SEQ ID NO:24); QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26);
LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID

NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and MHQPPQPLPPTVMFP (SEQ ID NO:34); an active analog thereof; and combinations thereof; with the proviso that the immunological regulator is not
 5 VESYVPLFP (SEQ ID NO:31).

As used herein, "a" or "an" means one or more (or at least one), such that combinations of active agents (i.e., active immunological regulators or blood cell differentiation promoters), for example, can be used in the compositions and methods of the invention. Thus, a composition that includes "a" polypeptide
 10 refers to a composition that includes one or more polypeptides.

"Amino acid" is used herein to refer to a chemical compound with the general formula: $\text{NH}_2\text{---CRH---COOH}$, where R, the side chain, is H or an organic group. Where R is organic, R can vary and is either polar or nonpolar (i.e., hydrophobic). The amino acids of this invention can be naturally occurring
 15 or synthetic (often referred to as nonproteinogenic). As used herein, an organic group is a hydrocarbon group that is classified as an aliphatic group, a cyclic group or combination of aliphatic and cyclic groups. The term "aliphatic group" means a saturated or unsaturated linear or branched hydrocarbon group. This term is used to encompass alkyl, alkenyl, and alkynyl groups, for example. The
 20 term "cyclic group" means a closed ring hydrocarbon group that is classified as an alicyclic group, aromatic group, or heterocyclic group. The term "alicyclic group" means a cyclic hydrocarbon group having properties resembling those of aliphatic groups. The term "aromatic group" refers to mono- or polycyclic aromatic hydrocarbon groups. As used herein, an organic group can be
 25 substituted or unsubstituted.

The terms "polypeptide" and "peptide" are used interchangeably herein to refer to a polymer of amino acids. These terms do not connote a specific length of a polymer of amino acids. Thus, for example, the terms oligopeptide, protein, and enzyme are included within the definition of polypeptide or peptide,
 30 whether produced using recombinant techniques, chemical or enzymatic synthesis, or naturally occurring. This term also includes polypeptides that have been modified or derivatized, such as by glycosylation, acetylation,

phosphorylation, and the like.

The following abbreviations are used throughout the application:

A = Ala = Alanine	T = Thr = Threonine
V = Val = Valine	C = Cys = Cysteine
5 L = Leu = Leucine	Y = Tyr = Tyrosine
I = Ile = Isoleucine	N = Asn = Asparagine
P = Pro = Proline	Q = Gln = Glutamine
F = Phe = Phenylalanine	D = Asp = Aspartic Acid
W = Trp = Tryptophan	E = Glu = Glutamic Acid
10 M = Met = Methionine	K = Lys = Lysine
G = Gly = Glycine	R = Arg = Arginine
S = Ser = Serine	H = His = Histidine

15 **Detailed Description of Preferred Embodiments of the Invention**

The inventors have found that colostrinin, at least one constituent (i.e., component) peptide thereof, and/or at least one active analog thereof (e.g., a peptide having an N-terminal sequence equivalent to an N-terminal sequence of at least one of the colostrinin constituent peptides) can be used to induce at least
 20 one cytokine (e.g., TNF- α , IFN- γ , IL-1, IL-2, IL-4, IL-6, I-10, IL-12). The cytokine can be either directly or indirectly induced. This can result in the modulation of an immune response or blood cell proliferation or differentiation (preferably, the promotion of blood cell proliferation, and more preferably, the promotion of blood cell proliferation and differentiation) *in vitro* and *in vivo*, in
 25 animals (including mammals such as humans).

Such immunological regulators and blood cell regulators are referred to herein as "active agents." Significantly, such agents can be administered alone or in various combinations to a patient (e.g., animals including humans) as a medication or dietary (e.g., nutrient) supplement in a dose sufficient to modulate
 30 one or more immune responses throughout the patient's body, in a specific tissue site, or in a collection of tissue sites.

Many nonspecific and specific immune responses are associated with

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leukocyte proliferation and differentiation. The overall immunological significance of the present invention can be, but is not limited to, the following: IFN- γ is a potent immunomodulator that is important for the development of the cytotoxic lymphocyte response (CTL). This immune response is considered to be very important in protecting humans and animals from a variety of bacterial, viral, parasitic, and fungal diseases. The fact that TNF- α is also induced is important because TNF- α is a major activator of macrophages, among other immune cells, which are important in host defense against infections. In addition, TNF- α has been shown to have activity against cancer, directly through its lytic activity and indirectly through macrophages. IL-10 is another important immune mediator that controls both IFN- γ and TNF- α production and action. Its production represent a negative feedback control for IFN- γ and TNF- α production. Another one of its hallmark activities is the control of antibody production during the humoral immune responses, which is certainly important in many types of infections. In addition to IL-10's immune activities, it also has been shown to play a role in the neuroendocrine system by modulating certain stress responses and immune responses. IL-10 has been shown to induce the production of corticotropin from pituitary cells. Corticotropin works downstream in the hypothalamic adrenal axis to induce glucocorticoid steroids that are inherently immunomodulatory. Like IL-10, the IL-4 is important in the development of B cell responses, which are the mediators of the humoral immune response. Finally, the IL-12 is an important IFN- γ inducer. Taken together these findings suggest that colostrinin and its component peptides have the ability to modulate via cytokine induction a variety of host-defense mechanisms mediated by macrophages and lymphocytes at the cellular and humoral immune level as well as the neuroendocrine system.

Thus, the methods and compositions of the present invention can be utilized to control immunological and blood cell differentiating activity. The active agents described herein can be used individually, in various combinations, or combined with other previously known or newly invented pharmacological agents, such as antioxidants. They can be used as adjuvants for existing vaccinations as well.

In a preferred embodiment, the present invention provides a method for modulating an immune response. Whether it be *in vivo* or *in vitro*, this method involves monitoring the level of at least one cytokine, which can be done by known methods, such as disclosed by Inglot et al., Arch. Immunol. Ther. Exp., 44, 215-224 (1996); Blach-Olszewska et al., Arch. Immunol. Ther. Exp., 45, 43-47 (1997); Piasecki et al., Arch. Immunol. Ther. Exp., 45, 109-117 (1997); Hughes et al., Int. J. Immunopharmacol., 17, 857-863 (1995); and Mishell et al., Selected Methods in Cellular Immunology, W.H. Freeman, 1980. Specific *in vitro* methods are described in the Examples Section.

In another preferred embodiment, the present invention provides a method for modulating blood cell proliferation (preferably, proliferation and differentiation). Whether it be *in vivo* or *in vitro*, this method involves monitoring the level of increase or decrease in the number of blood cells bearing a specific phenotypic marker (for differentiation, the types of cells formed are evaluated), as disclosed by Kim et al., Clin. Lab. Haematol., 20, 21-29 (1998); Grunwald et al., Methods Mol. Biol., 119, 443-454 (1999); Villas et al., Cell. Vis., 5, 56-61 (1998); and Gratama et al., Cytometry, 33, 166-178 (1998). Specific *in vitro* methods are described in the Examples Section.

The peptides described herein may be used for the proliferation and/or differentiation of other types of cells as well.

Colostrinin is composed of peptides, the aggregate of which has a molecular weight range between about 5.8 to about 26 kiloDaltons (kDa) determined by polyacrylamide gel electrophoresis. It has a greater concentration of proline than any other amino acid. Ovine colostrinin has been found to have a molecular weight of about 18 kDa and includes three non-covalently linked subunits having a molecular weight of about 6 kDa and has about 22 wt-% proline. Ovine colostrinin has also been shown to contain the following number of residues per subunit: lysine - 2; histidine - 1; arginine - 0; aspartic acid - 2; threonine - 4; serine - 3; glutamic acid - 6; proline - 11; glycine - 2; alanine - 0; valine - 5; methionine - 2; isoleucine - 2; leucine - 6; tyrosine - 1; phenylalanine - 3; and cysteine - 0.

Colostrinin has been found to include a number of peptides ranging from

3 amino acids to 22 amino acids or more. These can be obtained by various known techniques, including isolation and purification involving electrophoresis and synthetic techniques. The specific method of obtaining colostrinin and SEQ ID NO:31 is described in International Publication No. WO-A-98/14473. Using

5 HPLC and Edelman Degradation, over 30 constituent peptides of colostrinin have been identified, which can be classified into several groups: (A) those of unknown precursor; (B) those having a β -casein homologue precursor; (C) those having a β -casein precursor; and (D) those having an annexin precursor. These peptides are described in International Patent Application PCT/GB00/02128,

10 filed June 2, 2000, claiming priority to June 2, 1999, and can be synthesized according to the general method described in the Examples Section. These peptides (i.e., constituent peptides of colostrinin), which can be derived from colostrinin or chemically synthesized, include: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLPFQVQS

15 (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFPKLKVEVFPFP (SEQ ID NO:8); VVMEV (SEQ ID NO:9); SEQP (SEQ ID NO:10); DKE (SEQ ID NO:11); FPPPK (SEQ ID NO:12); DSQPPV (SEQ ID NO:13); DPPPPQS (SEQ ID NO:14); SEEMP

20 (SEQ ID NO:15); KYKLQPE (SEQ ID NO:16); VLPPNVG (SEQ ID NO:17); VYPFTGPIPN (SEQ ID NO:18); SLPQNILPL (SEQ ID NO:19); TQTPVVVPPF (SEQ ID NO:20); LQPEIMGVPKVKETMVPK (SEQ ID NO:21); HKEMPFPKY PVEPFTESQ (SEQ ID NO:22); SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23); SWMHQPP (SEQ ID NO:24);

25 QPLPPTVMFP (SEQ ID NO:25); PQSVLS (SEQ ID NO:26); LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27); AFLLYQE (SEQ ID NO:28); RGPFPILV (SEQ ID NO:29); ATFNRYQDDHGEEILKSL (SEQ ID NO:30); VESYVPLFP (SEQ ID NO:31); FLLYQEPVLGPVR (SEQ ID NO:32); LNF (SEQ ID NO:33); and MHQPPQPLPPTVMFP (SEQ ID NO:34).

30 These can be classified as follows: (A) those of unknown precursor include SEQ ID NOs:2, 6, 7, 8, 10, 11, 14, and 33; (B) those having a β -casein homologue precursor include SEQ ID NOs:1, 3, 4, 5, 9, 12, 13, 15, 16, 17, and

31; (C) those having a β -casein precursor include SEQ ID NOs:18 (casein amino acids 74-83), 19 (casein amino acids 84-92), 20 (casein amino acids 93-102), 21 (casein amino acids 103-120), 22 (casein amino acids 121-138), 23 (casein amino acids 139-156), 24 (casein amino acids 157-163), 25 (casein amino acids 164-173), 26 (casein amino acids 174-179), 27 (casein amino acids 180-201), 28 (casein amino acids 202-208), 29 (casein amino acids 214-222), 32 (casein amino acids 203-214), and 34 (casein amino acids 159-173); and (D) those having an annexin precursor include SEQ ID NO:30 (annexin amino acids 203-220).

10 For certain embodiments, a preferred group of such peptides does not include SEQ ID NO:31. A more preferred group of such peptides includes: MQPPPLP (SEQ ID NO:1); LQTPQPLLQVMMEPQGD (SEQ ID NO:2); DQPPDVEKPDLPFQVQS (SEQ ID NO:3); LFFFLPVVNVLP (SEQ ID NO:4); DLEMPVLPVEPFPPFV (SEQ ID NO:5); MPQNFYKLPQM (SEQ ID NO:6); VLEMKFPPPPQETVT (SEQ ID NO:7); LKPFKPKLKVEVFPFP (SEQ ID NO:8); VYPFTGPIP (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), HKEMPFPKYPVEPFOTESQ (SEQ ID NO:22), and combinations thereof.

20 The polypeptides of SEQ ID NOs:1-34 can be in their free acid form or they can be amidated at the C-terminal carboxylate group. The present invention also includes analogs of the polypeptides of SEQ ID NOs:1-34, which includes polypeptides having structural similarity with SEQ ID NOs:1-34. These peptides can also form a part of a larger peptide. An "analog" of a polypeptide includes at least a portion of the polypeptide, wherein the portion contains deletions or additions of one or more contiguous or noncontiguous amino acids, or containing one or more amino acid substitutions. An "analog" can thus include additional amino acids at one or both of the termini of the polypeptides listed above. Substitutes for an amino acid in the polypeptides of the invention are preferably conservative substitutions, which are selected from other members of the class to which the amino acid belongs. For example, it is well-known in the art of protein biochemistry that an amino acid belonging to a grouping of amino acids having a particular size or characteristic (such as

charge, hydrophobicity and hydrophilicity) can generally be substituted for another amino acid without substantially altering the structure of a polypeptide.

For the purposes of this invention, conservative amino acid substitutions are defined to result from exchange of amino acids residues from within one of the following classes of residues: Class I: Ala, Gly, Ser, Thr, and Pro (representing small aliphatic side chains and hydroxyl group side chains); Class II: Cys, Ser, Thr and Tyr (representing side chains including an -OH or -SH group); Class III: Glu, Asp, Asn and Gln (carboxyl group containing side chains); Class IV: His, Arg and Lys (representing basic side chains); Class V: Ile, Val, Leu, Phe and Met (representing hydrophobic side chains); and Class VI: Phe, Trp, Tyr and His (representing aromatic side chains). The classes also include related amino acids such as 3Hyp and 4Hyp in Class I; homocysteine in Class II; 2-aminoadipic acid, 2-aminopimelic acid, γ -carboxyglutamic acid, β -carboxyaspartic acid, and the corresponding amino acid amides in Class III; ornithine, homoarginine, N-methyl lysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutyric acid, homoarginine, sarcosine and hydroxylysine in Class IV; substituted phenylalanines, norleucine, norvaline, 2-aminooctanoic acid, 2-aminoheptanoic acid, statine and β -valine in Class V; and naphthylalanines, substituted phenylalanines, tetrahydroisoquinoline-3-carboxylic acid, and halogenated tyrosines in Class VI.

Preferably, active analogs of colostrinin and its constituent peptides include polypeptides having a relatively large number of proline residues. Because proline is not a common amino acid, a "large number" preferably means that a polypeptide includes at least about 15% proline (by number), and more preferably at least about 20% proline (by number). Most preferably, active analogs include more proline residues than any other amino acid. For certain embodiments, preferred group of such active analogs does not include SEQ ID NO:31.

As stated above, active analogs of colostrinin and its constituent peptides include polypeptides having structural similarity. Structural similarity is generally determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their

sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order.

Preferably, two amino acid sequences are compared using the Blastp program,

5 version 2.0.9, of the BLAST 2 search algorithm, available at

<http://www.ncbi.nlm.nih.gov/gorf/bl2.html>. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences

10 using the BLAST search algorithm, structural similarity is referred to as

"identity." Preferably, an active analog of colostrinin or its constituent peptides has a structural similarity to colostrinin or one or more of its constituent peptides (preferably, one of SEQ ID NOs:1-30) of at least about 70% identity, more preferably, at least about 80% identity, and most preferably, at least about 90%

15 identity.

Colostrinin or any combination of its peptide components or active analogs thereof can be derived (preferably, isolated and purified) naturally such as by extraction from colostrum or can be synthetically constructed using known peptide polymerization techniques. For example, the peptides of the invention

20 may be synthesized by the solid phase method using standard methods based on either t-butyloxycarbonyl (BOC) or 9-fluorenylmethoxy-carbonyl (Fmoc) protecting groups. This methodology is described by G.B. Fields et al. in

Synthetic Peptides: A User's Guide, W.M. Freeman & Company, New York, NY, pp. 77-183 (1992). Moreover, gene sequence encoding the colostrinin

25 peptides or analogs thereof can be constructed by known techniques such as expression vectors or plasmids and transfected into suitable microorganisms that will express the DNA sequences thus preparing the peptide for later extraction from the medium in which the microorganism are grown. For example, U.S.

Patent No. 5,595,887 describes methods of forming a variety of relatively small
30 peptides through expression of a recombinant gene construct coding for a fusion protein which includes a binding protein and one or more copies of the desired target peptide. After expression, the fusion protein is isolated and cleaved using

chemical and/or enzymatic methods to produce the desired target peptide.

The peptides used in the methods of the present invention may be employed in a monovalent state (i.e., free peptide or a single peptide fragment coupled to a carrier molecule). The peptides may also be employed as
5 conjugates having more than one (same or different) peptide fragment bound to a single carrier molecule. The carrier may be a biological carrier molecule (e.g., a glycosaminoglycan, a proteoglycan, albumin or the like) or a synthetic polymer (e.g., a polyalkyleneglycol or a synthetic chromatography support). Typically, ovalbumin, human serum albumin, other proteins, polyethylene
10 glycol, or the like are employed as the carrier. Such modifications may increase the apparent affinity and/or change the stability of a peptide. The number of peptide fragments associated with or bound to each carrier can vary, but from about 4 to 8 peptides per carrier molecule are typically obtained under standard coupling conditions.

15 For instance, peptide/carrier molecule conjugates may be prepared by treating a mixture of peptides and carrier molecules with a coupling agent, such as a carbodiimide. The coupling agent may activate a carboxyl group on either the peptide or the carrier molecule so that the carboxyl group can react with a nucleophile (e.g., an amino or hydroxyl group) on the other member of the
20 peptide/carrier molecule, resulting in the covalent linkage of the peptide and the carrier molecule. For example, conjugates of a peptide coupled to ovalbumin may be prepared by dissolving equal amounts of lyophilized peptide and ovalbumin in a small volume of water. In a second tube, 1-ethyl-3-(3-
25 dimethylamino-propyl)-carbodiimide hydrochloride (EDC; ten times the amount of peptide) is dissolved in a small amount of water. The EDC solution was added to the peptide/ovalbumin mixture and allowed to react for a number of hours. The mixture may then dialyzed (e.g., into phosphate buffered saline) to obtain a purified solution of peptide/ovalbumin conjugate. Peptide/carrier
30 molecule conjugates prepared by this method typically contain about 4 to 5 peptides per ovalbumin molecule.

The present invention also provides a composition that includes one or more active agents (i.e., colostrinin, at least one constituent peptide thereof, or

active analog thereof) of the invention and one or more carriers, preferably a pharmaceutically acceptable carrier. The methods of the invention include administering to, or applying to the skin of, a patient, preferably a mammal, and more preferably a human, a composition of the invention in an amount effective to produce the desired effect. The active agents of the present invention are formulated for enteral administration (oral, rectal, etc.) or parenteral administration (injection, internal pump, etc.). The administration can be via direct injection into tissue, interarterial injection, intervenous injection, or other internal administration procedures, such as through the use of an implanted pump, or via contacting the composition with a mucus membrane in a carrier designed to facilitate transmission of the composition across the mucus membrane such as a suppository, eye drops, inhaler, or other similar administration method or via oral administration in the form of a syrup, a liquid, a pill, capsule, gel coated tablet, or other similar oral administration method. The active agents can be incorporated into an adhesive plaster, a patch, a gum, and the like, or it can be encapsulated or incorporated into a bio-erodible matrix for controlled release.

The carriers for internal administration can be any carriers commonly used to facilitate the internal administration of compositions such as plasma, sterile saline solution, IV solutions or the like. Carriers for administration through mucus membranes can be any well-known in the art. Carriers for administration oral can be any carrier well-known in the art.

The formulations may be conveniently presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into the desired formulations.

Formulations suitable for parenteral administration conveniently include a sterile aqueous preparation of the active agent, or dispersions of sterile powders of the active agent, which are preferably isotonic with the blood of the

recipient. Isotonic agents that can be included in the liquid preparation include sugars, buffers, and sodium chloride. Solutions of the active agent can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions of the active agent can be prepared in water, ethanol, a polyol (such as glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, glycerol esters, and mixtures thereof. The ultimate dosage form is sterile, fluid, and stable under the conditions of manufacture and storage. The necessary fluidity can be achieved, for example, by using liposomes, by employing the appropriate particle size in the case of dispersions, or by using surfactants.

10 Sterilization of a liquid preparation can be achieved by any convenient method that preserves the bioactivity of the active agent, preferably by filter sterilization. Preferred methods for preparing powders include vacuum drying and freeze drying of the sterile injectible solutions. Subsequent microbial contamination can be prevented using various antimicrobial agents, for example, antibacterial, antiviral and antifungal agents including parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. Absorption of the active agents over a prolonged period can be achieved by including agents for delaying, for example, aluminum monostearate and gelatin.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as tablets, troches, capsules, lozenges, wafers, or cachets, each containing a predetermined amount of the active agent as a powder or granules, as liposomes containing the active agent, or as a solution or suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, or a draught. The amount of active agent is such that the dosage level will be effective to produce the desired result in the subject.

Nasal spray formulations include purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes. Formulations for rectal or vaginal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids. Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are

preferably adjusted to match that of the eye. Topical formulations include the active agent dissolved or suspended in one or more media such as mineral oil, DMSO, polyhydroxy alcohols, or other bases used for topical pharmaceutical formulations.

- 5 Useful dosages of the active agents can be determined by comparing their *in vitro* activity and the *in vivo* activity in animal models. Methods for extrapolation of effective dosages in mice, and other animals, to humans are known in the art; for example, see U.S. Patent No. 4,938,949.

- 10 The tablets, troches, pills, capsules, and the like may also contain one or more of the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; an excipient such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, fructose, lactose or aspartame; and a natural or artificial flavoring agent. When the unit dosage
- 15 form is a capsule, it may further contain a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials may be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules may be coated with gelatin, wax, shellac, or sugar and the like. A syrup or elixir may contain one or more of a sweetening
- 20 agent, a preservative such as methyl- or propylparaben, an agent to retard crystallization of the sugar, an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol, a dye, and flavoring agent. The material used in preparing any unit dosage form is
- 25 substantially nontoxic in the amounts employed. The active agent may be incorporated into sustained-release preparations and devices.

Examples

- 30 The invention will be further described by reference to the following detailed examples. The examples are meant to provide illustration and should not be construed as limiting the scope of the present invention. All peptides were dissolved in a balanced salt solution and/or DMSO.

Preparation of Peptides:

1. Wash pre-loaded resin with DMF (dimethylformamide), then drain completely.
2. Add 10 ml of 20% piperidine/DMF to resin. Shake for 5 minutes, then drain.
3. Add another 10 ml of 20% piperidine/DMF. Shake for 30 minutes.
4. Drain reaction vessel and wash resin with DMF four times. Then wash once with DCM (dichloromethanol). Check beads using the ninhydrin test - the beads should be blue.
5. The coupling step was carried out as follows:
 - a. Prepare the following solution: 1 mmole Fmoc (i.e. fluorenylmethyloxycarbonyl) amino acid 2.1 ml of 0.45 M HBTU/HOBT (1 mmol) (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole-H₂O) 348 µl of DIEA (2 mmol) (diisopropylethylamine); and
 - b. Add the solution to the resin and shake for a minimum of 30 minutes.
6. Drain reaction vessel and wash the resin again with DMF four times and with DCM once.
7. Perform the ninhydrin test: If positive (no colour) - proceed to step 2 and continue synthesis; If negative (blue colour) - return to step 5 and recouple the same Fmoc amino acid.
8. After the synthesis was complete, the peptide was cleaved from the resin with 5% H₂O, 5% phenol, 3% Thionisole, 3% EDT (ethanedithiol), 3% triisopropylsilane and 81% TFA for 2 hours.
9. After 2 hours, filter into cold MTBE (methyl t-butyl ether). The precipitated peptide was then washed twice with cold MTBE and dried under nitrogen gas.
10. The molecular weight of the synthesised peptides was checked by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectroscopy (LDMS), and the purity was checked by HPLC using a C-18, 300 Angstrom, 5 µm column.

Induction of Blood Cell Proliferation: The quantity of peripheral blood leukocyte (PBL) stimulation was determined by measuring the amount of ^3H -thymidine (1.0 to 2.0 μCi thymidine/culture) incorporated into triplicate cultures (4 x 10⁵ PBLs/culture) stimulated with colostrinin and its constituent peptides (CCP) for 72 hours. ^3H -thymidine was then added and allowed to incorporate for 24 hours. Staphylococcal enterotoxin A (SEA, also referred to as "super antigen"), a specific T cell mitogen, was used as a positive control and for comparative purposes. Colostrum and low and high iron containing baby formulas diluted 1:5 and 1:10 were also used in some experiments to determine the relative stimulatory activity of these products. Radioactivity was measured in a Matrix 9600 Direct Beta Counter. Six replicas of medium treated cultures were used to determine the mean background incorporated counts. The data is expressed as the mean ^3H -thymidine counts per minute (CPM) above background. Results of one out of a total of six experiments are shown below in Table 1.

It can be seen that colostrinin and its constituent peptides are excellent inducers of PBL proliferation. Active concentrations ranged from 100 $\mu\text{g/ml}$ to 0.1 $\mu\text{g/ml}$. Nine peptides and colostrinin and colostrum were tested. Certain peptides appeared to have greater activity than others with the maximum increase in proliferative activity being roughly 10 times above background. It appears that with many of the peptides, the active range of proliferation induction was present since concentrations as low as 0.1 $\mu\text{g/ml}$ still had potent activity. Some of the peptides had more activity than colostrinin alone. Another interesting finding is that colostrum appears to have roughly an equivalent amount of activity as colostrinin. SEA has the greatest activity and this is also not unexpected due to its classification as a super antigen. PBL proliferation is an important part of the immune response both for generating antigen reactive cells and induction of numerous modulating cytokines. In the newborn these processes are essential as a building block for development of an optimal immune response and provide a protective host defense barrier against diseases associated with the neonatal gut.

Table 1 - Effect of CCP on Fresh Human Leukocyte Cultures

	Peptide	Peptide Conc. µg/ml	Slide No.	Microscope 3 plus to 0	Mitogenic Activity CPM
5	SEQ ID NO:1	100	1	+++	1259
		10	2	++	4556
		1.0	3	+	4829
		0.1	4	+/-	3339
	SEQ ID NO:7	100	5	++	1383
		10	6	+	3478
		1.0	7	+/-	2290
		0.1	8	-	584
10	SEQ ID NO:8	100		-	2039
		10	9	-	1810
		1.0	10	+++	1527
		0.1	11	++	2177
	SEQ ID NO:3	100	ND	-	469
		10	ND	-	819
		1.0	ND	-	3323
		0.1	ND	-	86
15	SEQ ID NO:2	100	ND	-	29
		10	12	-	2989
		1.0	13	++	4809
		0.1	14	+/-	3578
	SEQ ID NO:4	100	15	+	2667
		10	16	+	4915
		1.0	ND	-	4050
		0.1	ND	-	3523
20	SEQ ID NO:5	100	ND	-	1762
		10	ND	-	3304
		1.0	ND	-	1938
		0.1	ND	-	1630
	SEQ ID NO:6	100	ND	-	748
		10	ND	-	3069
		1.0	ND	-	1375
		0.1	ND	-	1171
	SEQ ID NO:31	100	23	+++	2039
		10	24	++	200
		1.0	25	+	901
		0.1	26	-	1875
	Colostrinin	10	20	++	2470
		1.0	21	+	1614
		0.1	22	-	2535
	Colostrum	100	17	++	1094
		10	18	-	2991

	1.0	19	-	3320
	0.1	ND	-	2717
25	SEA	.02	ND	6554
	Control	27	-	461

ND = not done

+++ = strong induction of lymphoblasts and/or monocytes

30 ++ = medium induction of lymphoblasts and/or monocytes

+ = low induction of lymphoblasts and/or monocytes

+/- = some induction of lymphoblasts and/or monocytes

- = same as control

35 Mitogenic Activity = CPM above control as determined by 24-hour ³H-thymidine incorporation.

Cytokine studies: Colostrinin has previously been shown in the literature to induce IFN- γ and TNF- α , as has Val-Glu-Ser-Tyr-Val-Pro-Leu-Phe-Pro (SEQ ID NO:31), which is disclosed in International Publication No. WO-A-98/1447. Thus, studies were done to investigate the individual peptides.

Cytokine concentrations were also determined from cells following 72 hours of incubation with concentrations of colostrinin and its constituent peptides (CCP) ranging from 100 to 0.1 μ g/ml, and colostrum and high- or low-iron baby formula (Enfamil) at various dilutions. Supernatant fluids were then subjected to enzyme-linked immunosorbent assay (ELISA) for the following commercially available cytokines: interferon-gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin (IL)-4, IL-6, IL-10, and IL-12.

Table 2 represents the results of approximately 250 single assays. More specifically, in these studies it was found that many of the peptides including colostrinin induced IFN- γ and that the data corresponds with ³H-thymidine incorporation (Tables 1 and 3). Interestingly the maximum cytokine inducing activity of many of the peptides was not diluted out until the 1.0 or 0.1 μ g/ml concentrations of peptide were used (Shaded numbers in Table 2), or in the case of IFN- γ and TNF- α induction by SEQ ID NO:31 and SEQ ID NO:1, 0.1 μ g/ml rather than higher concentrations. This finding may be consistent with a phasic response like those of hormones or of toxicity present in higher concentrations.

The ability to induce IFN- γ by some of the peptides decayed over time.

For example, SEQ ID NO:31 at 0.1 µg/ml at the beginning of the studies induced 324 pg IFN-γ/ml and in the last experiments induced no detectable levels. Although the peptides lost the IFN-γ inducing activity over a period of four months when stored in solution, some of the peptides were still able to induce TNF-α, IL-6, and IL-12, but the levels produced were somewhat lower than in the earlier studies. In contrast, induction of TNF-α and IL-10 by colostrinin and colostrum was still very high at this time. Thus, the complexed peptides making up colostrinin and colostrum may be more stable and/or combinations of peptides in colostrinin and colostrum may be more potent.

Additional factors that may account for the variations of the peptides in these studies include: 1) natural variations in the immune state of the individuals donating the leukocytes, 2) the possibility that aggregation occurred in samples stored in PBS, thus reducing in effective number of molecules able to react, and 3) the possibility that the individual peptides may be subject to oxidative damage or some other inactivating process. The fact that the peptide, SEQ ID NO:8, which still induced IFN-γ in the last experiment (Example 3) was stored in 33% DMSO suggests an oxidative process or aggregation problem may be responsible for loss, or reduction of inducing activity in peptide samples stored in phosphate buffered saline (PBS). However, the samples in PBS appeared to be in solution at the time of the induction experiments.

	PEPTIDE (Exp. #)	PEPTIDE CONCENTRATION (mg/ml)	IFN γ (pg/ml)	TNF- α (pg/ml)	IL-10 (pg/ml)
10	Example 1				
	SEQ ID NO:1	100	54	478	168
		10	526	>1000	940
		1	584	>1000	1070
		0.1	236	722	696
15	SEQ ID NO:7	100	317	>1000	998
		10	409	>1000	1134
20	SEQ ID NO:8	100	419	>1000	860
		10	775	>1000	1643
		1	877	>1000	2223
		0.1	642	>1000	1350
25	SEQ ID NO:3	1	809	>1000	1611
		0.1	206	802	611
30	SEQ ID NO:2	100	372	>1000	754
		10	410	>1000	1063
		1	826	>1000	2092
		0.1	259	>1000	596
	SEQ ID NO:4	10	794	>1000	1494
		1	723	>1000	1765
35	SEQ ID NO:5	100	559	>1000	756
		10	626	>1000	1158
	SEQ ID NO:6	100	91	718	302
		10	621	>1000	1203
40	SEQ ID NO:31	100	371	804	4234
		10	107	370	1834
		1	118	651	242
		0.1	324	>1000	356

	Colistrinin	10	888	>1000	1515
		1	878	>1000	1150
		0.1	156	760	451
5	Raw	100	807	>1000	857
	Colostrum	10	530	>1000	1074
		1	934	>1000	1645
		0.1	192	848	391
10	Control		4	52	0
	SEA		902	>1000	4676
15	Example 2				
20	SEQ ID NO:18	100	4	24	36
	SEQ ID NO:19	10	6	65	76
		1	463	>1000	502
25	SEQ ID NO:20	100	9	30	21
		10	31	118	101
	SEQ ID NO:22	100	535	>1000	524
		10	539	985	409
30		1	649	>1000	460
		0.1	147	636	207
	SEQ ID NO:1	100	9	92	108
		10	14	99	129
35		1	287	728	292
		0.1	576	>1000	397
	SEQ ID NO:7	100	762.9	>1000	639
40	SEQ ID NO:2	100	980	>1000	646
		10	828	>1000	651
		1	914	>1000	1093
		0.1	281	685	348

	Enfamil Low Iron	1:5 1:10	101 167	305 406	24 443
5	Enfamil with Iron	1:5 1:10	24 10	528 320	136 702
	Control		7	248	180
10	SEA		901	>1000	2806
Example 3					
15	SEQ ID NO:1	100 10	6 4	110 ND	0 ND
	SEQ ID NO:7	1 0.1	9 6	57 ND	0 ND
20	SEQ ID NO:8	10 1	8 288	20 ND	0 ND
	SEQ ID NO:5	100	3	0	0
25	Raw Colostrum	100 10 1 0.1	5 15 0 0	11 520 ND ND	0 569 ND ND
30	Colostrinin	10 1 0.1	0 18 1	>1000 910 ND	3662 1839 ND
35	SEQ ID NO:31	10 1 0.1	0 0 0	11 90 ND	0 0 ND
40	SEQ ID NO:22	100 10 1 0.1	0 0 0 0	120 60 7 ND	77.6 0 0 ND

	Enfamil Low Iron	1:5	25	339	51
5	Enfamil with Iron	1:5	0	452	51
	Control		0	0	0
10	SEA		700	>1000	2971
Example 4					
	SEQ ID NO:1	100	0	73.3	0
15	SEQ ID NO:2	1	0	0	0
	Colostrinin	10	0	1790	6.9
		1	0	1813	0
20		0.1	ND	ND	ND
	Raw Colostrum	100	0	1834	4.0
		10	0	31.2	0
		1	ND	ND	ND
25	Control		0	28.4	0
	SEA		3.5	1927	13.4

Table 2. (cont.) Cytokines induced in human leukocyte cultures stimulated with CCP, colostrum or commercial milk formulas.

5	PEPTIDE	PEPTIDE	IL-4	IL-6	IL-12
	(Exp. #)	CONCENTRATION (mg/ml)	(pg/ml)	(pg/ml)	(pg/ml)
<hr/>					
10	Example 1				
	SEQ ID NO:1	100	0	235.4	0
		10	0	934.8	0
		1	0	675.3	0
		0.1	0	497.1	0
15	SEQ ID NO:7	100	0	291.3	0
		10	0	645.4	0
20	SEQ ID NO:8	100	0	1076	0
		10	0	1024	0
		1	0	1013	0
		0.1	0	533.6	0
25	SEQ ID NO:3	1	0	620.5	0
		0.1	0	107	0
30	SEQ ID NO:2	100	0	258.6	0
		10	0	551.3	0
		1	0	1205	0
		0.1	0	325	0
35	SEQ ID NO:4	10	0	1718	0
		1	0	744.4	0
40	SEQ ID NO:5	100	0	98.2	0
		10	0	750	0
	SEQ ID NO:6	100	0	63.3	0
		10	0	864.5	0

	SEQ ID NO:31	100	1.4	1489	0
		10	0	836.3	0
		1	0.4	489.9	0
		0.1	2.4	1635	0
5	Colostrinin	10	0	1832	0
		1	1.9	1915	0
		0.1	0.4	430.1	0
10	Raw Colostrum	100	0	1879	0
		10	0	602.2	0
		1	0	1055	0
		0.1	5.0	187.2	0
15	Control		0	13.5	0
	SEA		4	1704	0
20	<u>Example 2</u>				
25	SEQ ID NO:18	100	0	142.4	0
	SEQ ID NO:19	10	0	549.7	0
		1	33.8	1552	0
	SEQ ID NO:20	100	0	50	0
		10	0.4	105.9	0
30	SEQ ID NO:22	100	41.5	808.6	0
		10	32.7	503.2	0
		1	30.1	1005	0
		0.1	17.8	396.4	0
35	SEQ ID NO:1	100	0	1471	0
		10	3.5	96.5	5.7
		1	26.6	626.6	0
		0.1	47.6	1385	0
40	SEQ ID NO:7	100	24.5	1546	0
	SEQ ID NO:2	100	22.5	1292	33.5
		10	19.9	1516	0

		1	10.1	1886	9.9
		0.1	29.1	478.3	2.2
5	Enfamil	1:5	0.9	1757	0
	Low Iron	1:10	4.0	1958	0
	Enfamil with Iron	1:5	0	1909	0
		1:10	0	ND	0
10	Control		0	183.5	0
	SEA		62.5	1769	54.8
15	Example 3				
	SEQ ID NO:1	100	0	942.5	0
		10	ND	ND	ND
20	SEQ ID NO:7	1	0	32.9	0
		0.1	ND	ND	ND
	SEQ ID NO:8	10	0	18.5	4.0
		1	ND	ND	ND
25	SEQ ID NO:5	100	0	0	0
30	Raw	100	0	0	0
	Colostrum	10	0	1853	1.6
		1	ND	ND	ND
		0.1	ND	ND	ND
35	Colostrinin	10	0	2009	17.6
		1	0	1861	7.5
		0.1	ND	ND	ND
	SEQ ID NO:31	10	0	16.8	18.7
		1	0	722.9	0
		0.1	ND	ND	ND
40	SEQ ID NO:22	100	6.0	1630	0
		10	0	46.7	0
		1	0	0	0

		0.1	ND	ND	ND
	Enfamil Low Iron	1:5	0	1913	0
5	Enfamil with Iron	1:5	0.4	1953	0
	Control		0	0	0
10	SEA		16.8	866.2	0

15 *SEQ ID NOs:1-8 and 31, Raw Colostrum, and Colostrinin were reconstituted on the same day.

*SEQ ID NOs:18, 19, 20, and 22 were reconstituted on the same day.

20 The relative abilities of the various peptides to induce cytokines are shown in Table 3. The peptides were ranked according to their abilities to induce the indicated cytokine by first comparing the raw numbers at the 0.1 µg/ml concentration followed by 1.0 µg/ml concentrations and then higher concentrations, i.e., 10 and 100 µg/ml. It can be noted that SEQ ID NOs:1, 8, 3, 2, and 31 were the best overall inducers in almost all cytokine and blood cell proliferation experiments. Peptides SEQ ID NOs:7, 4, and 5 were generally less effective as inducers. Colostrinin and colostrum ranked generally in the middle, however, only 1:5 and 1:10 dilutions of colostrum were used, thus actual comparison are not accurate since specific protein species present and their concentrations were not determined for colostrum. It is important to note, however, that colostrum contained substances that could induce cytokines in a similar fashion to colostrinin and its component peptides.

30 When the colostrinin constituent peptides having a β-casein precursor (SEQ ID NOs: 18, 19, 20, and 22) were compared to the initially tested SEQ ID NOs:1-8 and 31, the latter were better inducers. SEQ ID NO:22 was generally the best inducer of those peptides having a β-casein precursor. It was also found that Enfamil low iron baby formula induced higher levels of cytokines than the Enfamil high iron formula.

**Table 3. Relative abilities of the various peptides to induce cytokines
and proliferation**

		Ex. 1	Ex. 2	Ex. 1	Ex. 1	Ex. 1	Ex. 2	Ex. 1
5	Rank	IFN- γ	IFN- γ	Micro. Resp.	Prolif. Resp.	TNF- α	TNF- α	IL-10
	1	SEQ ID NO:8	SEQ ID NO:1	SEQ ID NO:8	SEQ ID NO:2	SEQ ID NO:2**	SEQ ID NO:2	SEQ ID NO:8
	2	SEQ ID NO:31	SEQ ID NO:2	SEQ ID NO:2	SEQ ID NO:1	SEQ ID NO:8	SEQ ID NO:1	SEQ ID NO:1
	3	SEQ ID NO:2	SEQ ID NO:7	SEQ ID NO:31	SEQ ID NO:4	SEQ ID NO:31	SEQ ID NO:7	SEQ ID NO:3
10	4	SEQ ID NO:1	SEQ ID NO:22	SEQ ID NO:1	Colostrum	Colostrum	SEQ ID NO:22	SEQ ID NO:2
	5	SEQ ID NO:3	SEQ ID NO:19	SEQ ID NO:7	Colostrinin	Colostrinin	SEQ ID NO:19	Colostrinin
	6	Colistrinin	SEQ ID NO:20	Colostrinin	SEQ ID NO:8	SEQ ID NO:3	SEQ ID NO:20	Colostrum
	7	Colustrum	SEQ ID NO:18	Colostrum	SEQ ID NO:31	SEQ ID NO:1	SEQ ID NO:18	SEQ ID NO:31
	8	SEQ ID NO:4		SEQ ID NO:3	SEQ ID NO:5	SEQ ID NO:5		SEQ ID NO:4
15	9	SEQ ID NO:5		SEQ ID NO:4	SEQ ID NO:6	SEQ ID NO:7	Low Enfamil	SEQ ID NO:7
	9	SEQ ID NO:6		SEQ ID NO:5	SEQ ID NO:7	SEQ ID NO:4	High Enfamil	SEQ ID NO:5
	10	SEQ ID NO:7		SEQ ID NO:6	SEQ ID NO:3	SEQ ID NO:6		SEQ ID NO:6

* SEQ ID NO:7 < 2 fold difference in titer

** All good inducers

*** No difference in titer

5	Rank	Ex. 2 IL-10	Ex. 1 IL-4	Ex. 2 IL-4	Ex. 1 IL-6	Ex. 2 IL-6	Ex. 1 IL-12	Ex. 2 IL-12
	1	SEQ ID NO:2	Colostrum	SEQ ID NO:1	SEQ ID NO:31	Control	All neg.	SEQ ID NO:2
	2	SEQ ID NO:7	Colostrinin	SEQ ID NO:2	SEQ ID NO:8			SEQ ID NO:1
10	3	SEQ ID NO:1	SEQ ID NO:31	SEQ ID NO:22	SEQ ID NO:1			
	4	SEQ ID NO:19		SEQ ID NO:19	Colostrinin			
	5	SEQ ID NO:22		SEQ ID NO:7	SEQ ID NO:2			
	6	SEQ ID NO:20		Low Enfamil	Colostrum			
	7	SEQ ID NO:18			SEQ ID NO:3			
15	8				SEQ ID NO:4			
	9	Low Enfamil			SEQ ID NO:6			
	9	High Enfamil			SEQ ID NO:5			
	10				SEQ ID NO:7			
20		* SEQ ID NO:7 < 2 fold difference in titer ** All good inducers *** No difference in titer						

Although the invention has been disclosed with reference to its preferred embodiments, from reading this description those of skill in the art may appreciate changes and modification that may be made which do not depart from the scope and spirit of the invention as described above and claimed hereafter. All references, patents, and patent applications cited herein are incorporated herein by reference in their entirety as if individually incorporated.

10 Sequence Listing Free Text

The following are all synthetic peptide sequences.

	SEQ ID NO:1	MQPPPLP
	SEQ ID NO:2	LQTPQPLLQVMMEPQGD
	SEQ ID NO:3	DQPPDVEKPDLPFQVQS
15	SEQ ID NO:4	LFFFLPVVNVLP
	SEQ ID NO:5	DLEMPVLPVEPFPPFV
	SEQ ID NO:6	MPQNFYKLPQM
	SEQ ID NO:7	VLEMKFPPPPQETVT
	SEQ ID NO:8	LKPFPPKLKVEVFPPF
20	SEQ ID NO:9	VVMEV
	SEQ ID NO:10	SEQP
	SEQ ID NO:11	DKE
	SEQ ID NO:12	FPPPK
	SEQ ID NO:13	DSQPPV
25	SEQ ID NO:14	DPPPPQS
	SEQ ID NO:15	SEEMP
	SEQ ID NO:16	KYKLQPE
	SEQ ID NO:17	VLPPNVG
	SEQ ID NO:18	VYPFTGPIPN
30	SEQ ID NO:19	SLPQNILPL
	SEQ ID NO:20	TQTPVVVPPF
	SEQ ID NO:21	LQPEIMGVPPKVKETMVPK

	SEQ ID NO:22	HKEMPFPKYPVEPFTESQ
	SEQ ID NO:23	SLTLTDVEKLHLPLPLVQ
	SEQ ID NO:24	SWMHQPP
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10	SEQ ID NO:31	VESYVPLFP
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	SEQ ID NO:34	MHQPPQPLPPTVMFP

002F80" T0B T4950

We claim:

1. A method of inducing a cytokine in a cell, the method comprising contacting the cell with an immunological regulator under conditions effective to induce a cytokine, wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34), an active analog thereof, and combinations thereof, with the proviso that the immunological regulator is not VESYVPLFP (SEQ ID NO:31).
2. The method of claim 1 wherein the cell is present in a cell culture, a tissue, an organ, or an organism.
3. The method of claim 1 wherein the cell is a mammalian cell.
4. The method of claim 3 wherein the cell is a human cell.
5. The method of claim 1 wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD

(SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3),
 LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPFV (SEQ ID NO:5),
 MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7),
 LKPFPKLKVEVFPEP (SEQ ID NO:8), VYPFTGPIPN (SEQ ID NO:18),
 5 SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20),
 HKEMPFKYPVEPFTESQ (SEQ ID NO:22), and combinations thereof.

6. A method for modulating an immune response in a cell, the method
 comprising contacting the cell with an immunological regulator under
 10 conditions effective to induce a cytokine, wherein the immunological regulator
 is selected from the group of MQPPPLP (SEQ ID NO:1),
 LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS
 (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPFV
 (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT
 15 (SEQ ID NO:7), LKPFPKLKVEVFPPF (SEQ ID NO:8), VVMEV (SEQ ID
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 25 NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID
 NO:30), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and
 MHQPPQPLPPTVMFP (SEQ ID NO:34), an active analog thereof, and
 combinations thereof, with the proviso that the immunological regulator is not
 VESYVPLFP (SEQ ID NO:31).

30

7. The method of claim 6 wherein the cell is present in a cell culture, a
 tissue, an organ, or an organism.

8. The method of claim 6 wherein the cell is a mammalian cell.
9. The method of claim 8 wherein the cell is a human cell.
10. The method of claim 6 wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDQLQPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPEP (SEQ ID NO:8), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), and combinations thereof.
11. A method for modulating an immune response in a patient, the method comprising administering to the patient an immunological regulator under conditions effective to induce a cytokine, wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDQLQPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPPF (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and

MHQPPQPLPPTVMFP (SEQ ID NO:34), an active analog thereof, and combinations thereof, with the proviso that the immunological regulator is not VESYVPLFP (SEQ ID NO:31).

- 5 12. The method of claim 11 wherein the immunological regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7),
10 LKPFPKLKVEVFPEP (SEQ ID NO:8), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), and combinations thereof.
13. The method of claim 11 wherein the immunological regulator is
15 administered as part of a dietary supplement.
14. The method of claim 11 wherein the immunological regulator is administered topically.
- 20 15. The method of claim 11 wherein the patient is an animal.
16. The method of claim 15 wherein the patient is a human.
17. The method of claim 11 wherein the immune response is a specific
25 immune response.
18. The method of claim 11 wherein the immune response is a nonspecific immune response.
- 30 19. The method of claim 11 wherein the immune response is the interferon response or antibody production.

20. A method for modulating blood cell proliferation, the method comprising contacting blood cells with a blood cell regulator selected from the group of colostrinin, a constituent peptide thereof, an analog thereof, and combinations thereof, under conditions effective to change the number of blood cells.
21. The method of claim 20 wherein the blood cells are present in a cell culture or an organism.
22. The method of claim 20 wherein the blood cells are mammalian cells.
23. The method of claim 22 wherein the blood cells are human cells.
24. The method of claim 22 wherein the blood cells are increased in number.
25. The method of claim 24 wherein the blood cells are differentiated.
26. The method of claim 22 wherein the blood cell regulator is a constituent peptide of colostrinin.
27. The method of claim 26 wherein the blood cell regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP

(SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), VESYVPLFP (SEQ ID NO:31), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34),
 5 an active analog thereof, and combinations thereof.

28. The method of claim 27 wherein the blood cell regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD
 10 (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPFV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPPFKLKVEVFPEP (SEQ ID NO:8), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20),
 15 HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), and combinations thereof.

29. A method for modulating blood cell proliferation in a patient, the method comprising administering to the patient a blood cell regulator selected from the group of colostrinin, a constituent peptide thereof, an analog thereof,
 20 and combinations thereof, under conditions effective to change the number of blood cells.

30. The method of claim 29 wherein the patient is a human.

25 31. The method of claim 29 wherein the blood cells are increased in number.

32. The method of claim 31 wherein the blood cells are differentiated.

33. The method of claim 29 wherein the blood cell regulator is a constituent
 30 peptide of colostrinin.

34. The method of claim 33 wherein the blood cell regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPPFKLKVEVFPPF (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24), QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), VESYVPLFP (SEQ ID NO:31), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34), an active analog thereof, and combinations thereof.

35. The method of claim 34 wherein the blood cell regulator is selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPV (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPPFKLKVEVFPEP (SEQ ID NO:8), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), and combinations thereof.

36. A cytokine-inducing composition comprising a pharmaceutical carrier and an active agent selected from the group of MQPPPLP (SEQ ID NO:1), LQTPQPLLQVMMEPQGD (SEQ ID NO:2), DQPPDVEKPDLPFQVQS (SEQ ID NO:3), LFFFLPVVNVLP (SEQ ID NO:4), DLEMPVLPVEPFPPV

- (SEQ ID NO:5), MPQNFYKLPQM (SEQ ID NO:6), VLEMKFPPPPQETVT (SEQ ID NO:7), LKPFPKLKVEVFPFP (SEQ ID NO:8), VVMEV (SEQ ID NO:9), SEQP (SEQ ID NO:10), DKE (SEQ ID NO:11), FPPPK (SEQ ID NO:12), DSQPPV (SEQ ID NO:13), DPPPPQS (SEQ ID NO:14), SEEMP
- 5 (SEQ ID NO:15), KYKLQPE (SEQ ID NO:16), VLPPNVG (SEQ ID NO:17), VYPFTGPIPN (SEQ ID NO:18), SLPQNILPL (SEQ ID NO:19), TQTPVVVPPF (SEQ ID NO:20), LQPEIMGVPKVKETMVPK (SEQ ID NO:21), HKEMPFPKYPVEPFTESQ (SEQ ID NO:22), SLTLTDVEKLHLPLPLVQ (SEQ ID NO:23), SWMHQPP (SEQ ID NO:24),
- 10 QPLPPTVMFP (SEQ ID NO:25), PQSVLS (SEQ ID NO:26), LSQPKVLPVPQKAVPQRDMPIQ (SEQ ID NO:27), AFLLYQE (SEQ ID NO:28), RGPFPILV (SEQ ID NO:29), ATFNRYQDDHGEEILKSL (SEQ ID NO:30), FLLYQEPVLGPVR (SEQ ID NO:32), LNF (SEQ ID NO:33), and MHQPPQPLPPTVMFP (SEQ ID NO:34), an active analog thereof, and
- 15 combinations thereof, with the proviso that the active agent is not VESYVPLFP (SEQ ID NO:31).

**USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND
ANALOGS THEREOF FOR INDUCING CYTOKINES**

5

Abstract of the Disclosure

The present invention discloses a use of colostrinin, a constituent peptide thereof, and/or an analog thereof as an immunological regulator and as a blood cell regulator in animals including humans.

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30 Application, Washington, D.C. 20231,

By: 

Name: Louise M. Guggisberg

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): G. John Stanton et al.)
Serial No.: unknown)
Filed: herewith on August 17, 2000)

Group Art Unit: unknown
Examiner: unknown

For: **USE OF COLOSTRININ, CONSTITUENT PEPTIDES THEREOF, AND
ANALOGS THEREOF FOR INDUCING CYTOKINES**

COMMUNICATION

Commissioner for Patents
ATTN: BOX SEQUENCE
Washington, D.C. 20231

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Date of Deposit: 17 August 2000
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By: 
Name: Louise M. Guggisberg

Respectfully submitted,

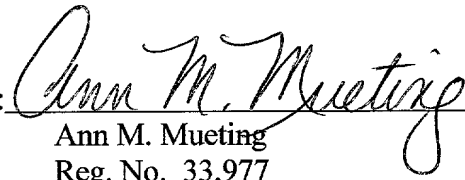
G. John Stanton et al.,

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Figure 1 displays 12 Western blot images showing protein levels of various markers in H1299 cells treated with 100 nM NVP-BAW298 for 24 hours. The blots are arranged in two columns of six. The left column shows: 1) GAPDH (loading control), 2) p-ERK1/2 (T202/Y204), 3) ERK1/2 (total), 4) p-PI3K (S473), 5) PI3K (total), and 6) p-Akt (S473). The right column shows: 7) Akt (total), 8) p-mTOR (S2448), 9) mTOR (total), 10) p-p70S6 (S2448), 11) p4E (total), and 12) p-p70S6 (S2448). Each blot has lanes for control (C), NVP-BAW298 (N), and a combination of NVP-BAW298 with either LY294002 (LY) or SB415286 (SB). Molecular weight markers are indicated on the right of each blot.

Figure 1 displays 12 Western blot images showing protein levels of various markers in H1299 cells treated with 100 nM NVP-BAW298 for 24 hours. The blots are arranged in two columns of six. The left column shows: 1) GAPDH (loading control), 2) p-ERK1/2 (T202/Y204), 3) ERK1/2 (total), 4) p-PI3K (S473), 5) PI3K (total), and 6) p-Akt (S473). The right column shows: 7) Akt (total), 8) p-mTOR (S2448), 9) mTOR (total), 10) p-p70S6 (S2448), 11) p4E (total), and 12) p-p70S6 (S2448). Each blot has lanes for control (C), NVP-BAW298 (N), and a combination of NVP-BAW298 with either LY294002 (LY) or SB415286 (SB). Molecular weight markers are indicated on the right of each blot.

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Figure 1 displays 12 Western blot images showing protein levels of various markers in H1299 cells treated with 100 nM NVP-BAW298 for 24 hours. The blots are arranged in two columns of six. The left column shows: 1) GAPDH (loading control), 2) p-ERK1/2 (T202/Y204), 3) ERK1/2 (total), 4) p-PI3K (S473), 5) PI3K (total), and 6) p-Akt (S473). The right column shows: 7) Akt (total), 8) p-mTOR (S2448), 9) mTOR (total), 10) p-p70S6 (S2448), 11) p4E (total), and 12) p-p70S6 (S2448). Each blot has lanes for control (C), NVP-BAW298 (N), and a combination of NVP-BAW298 with either LY294002 (LY) or SB415286 (SB). Molecular weight markers are indicated on the right of each blot.

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<220>
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peptide

<400> 25
Gln Pro Leu Pro Pro Thr Val Met Phe Pro
1 5 10

<210> 26
<211> 6
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<213> Artificial Sequence

<220>
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peptide

<400> 26
Pro Gln Ser Val Leu Ser
1 5

<210> 27
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<220>
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peptide

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Val Glu Ser Tyr Val Pro Leu Phe Pro
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<210> 32
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<220>
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peptide

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1 5 10

<210> 33
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<220>
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peptide

<400> 33
Leu Asn Phe
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<210> 34
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<220>
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peptide

<400> 34
Met His Gln Pro Pro Gln Pro Leu Pro Pro Thr Val Met Phe Pro
1 5 10 15